INFECTION AND IMMUNITY, Apr. 1986, p. 90–95
0019-9567/86/040090-06$02.00/0

Vol. 52, No. 1

Physiological Effects of a Bactericidal Protein from Human Polymorphonuclear Leukocytes on *Pseudomonas aeruginosa*

CAROLYN J. HOVDE* AND BEULAH H. GRAY

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

Received 23 September 1985/Accepted 10 December 1985

The physiological changes seen in *Pseudomonas aeruginosa* after exposure to a bactericidal protein (BP) from the granules of human polymorphonuclear leukocytes were studied. It was demonstrated, using radiolabeled proline or leucine, that both the rate of cellular uptake and amino acid incorporation into trichloroacetic acid-insoluble material were markedly decreased immediately after exposure to BP. The rate of O_2 consumption by *P. aeruginosa* was decreased immediately after exposure to BP and continued to decline exponentially until it ceased completely 30 min after exposure to BP. In the presence of 30 mM $CaCl_2$ or $MgCl_2$, bacteria were protected from death due to BP and respiration rates were unaffected. The cellular ATP pool of *P. aeruginosa* remained constant for up to 2 h after exposure to BP. Membrane depolarization was measured by the influx of the lipophilic anion thiocyanate. It was shown that the cytoplasmic membrane of *P. aeruginosa* was partially depolarized after exposure to BP. Purified BP killed 95% of 5×10^6 CFU of *P. aeruginosa* at a concentration of 60 to 100 ng of protein per ml. Although the concentration of bacteria and BP varied with each type of experiment, the BP/bacteria ratio required to cause a 95 to 99% loss in viability remained constant. We propose that cytoplasmic membrane depolarization is the biochemical lesion responsible for the other physiological changes seen and ultimately for the death of *P. aeruginosa* induced by BP.

Over the past 20 years, *Pseudomonas aeruginosa* has assumed an increasingly prominent role as the etiological agent in serious infections among immunocompromised, hospitalized patients (10). It is now responsible for the highest mortality rates among the opportunistic gram-negative infections. One of the most important host-protective mechanisms against *P. aeruginosa* infection is the polymorphonuclear leukocyte (PMN). The susceptibility of patients to infection with this bacterium can often be attributed to a failure of their PMN to ingest and kill bacteria (12, 18).

The array of microbicidal mechanisms available to PMN, traditionally divided into oxygen-dependent and oxygen-independent systems, has been recently reviewed (1, 5, 14). We have shown that the oxygen-independent bactericidal mechanisms of PMN from patients with chronic granulomatous disease is sufficiently active to kill *P. aeruginosa* with the same efficiency as do PMN from normal healthy individuals (4). When partially purified from the granule fraction of normal PMN, the bactericidal activity against *P. aeruginosa* was relatively independent of pH over the 5.0 to 7.0 range and was inactivated by trypsin (N. K. Henry, Ph.D. thesis, University of Minnesota, Minneapolis, 1980).

A major factor contributing to the high mortality associated with infections caused by *P. aeruginosa* is resistance of the organism to a broad range of antibiotics. Resistance often develops even when aggressive multiple drug therapy is used. It is impressive that this highly versatile organism, with the potential to become a devastating pathogen, is easily and efficiently destroyed by the normal PMN found in healthy individuals. For this reason, it was of interest to explore the molecular mechanism by which PMN kill *P. aeruginosa*.

Our laboratory has purified a bactericidal protein (BP) from the cytoplasmic granules of normal human PMN which has remarkable potency against *P. aeruginosa*. In this paper we report the physiological changes seen in *P. aeruginosa* after exposure to BP. It was demonstrated that the rate of amino acid uptake, amino acid incorporation, and respiration all decreased immediately upon exposure to BP. It was also shown that BP depolarized the bacterial cytoplasmic membrane. We believe that cytoplasmic membrane damage is the biochemical lesion responsible for the other physiological changes seen and ultimately for the death of *P. aeruginosa*.

MATERIALS AND METHODS

Microorganisms. *P. aeruginosa* type I was isolated from a patient at the University of Minnesota Hospitals, *Escherichia coli* B, a rough strain, was obtained from Paul Quie, University of Minnesota, and *P. aeruginosa* ATCC 27312 and *Proteus mirabilis* ATCC 25608 were purchased from the American Type Culture Collection. Bacteria were maintained on blood agar plates. To obtain exponential growth, cells were grown overnight in brain heart infusion broth, transferred to fresh brain heart infusion broth, and grown for 3 to 4 h. Unless otherwise stated, bacteria were harvested by centrifugation, washed in 0.08 M citrate-phosphate (CP) buffer (pH 5.6), suspended in a mixture of CP and nutrient broth (NB), and adjusted by optical density measurement to an appropriate cell concentration. Exact cell numbers were previously determined by viable plate counts on nutrient agar.

Isolation of BP. BP was purified from a mixed population of cytoplasmic granules isolated from normal, platelet-free PMN at 82 to 98% purity. A manuscript (C. J. Hovde and B. H. Gray, detailing the purification and characterization of BP is in preparation. Briefly, the cytoplasmic granules from 10^{10} PMN were extracted by sonication in 0.01 N HCl. The extract was applied to a column of Orange A Matrex Gel (Amicon Corp., Lexington, Mass.), and bound proteins were eluted with a gradient of 0.1 to 1.6 M NaCl in CP buffer, pH 5.6. Fractions containing bactericidal activity toward *P. aeruginosa* were applied to a Bio-Rex 70 (Bio-Rad Labora-

* Corresponding author.

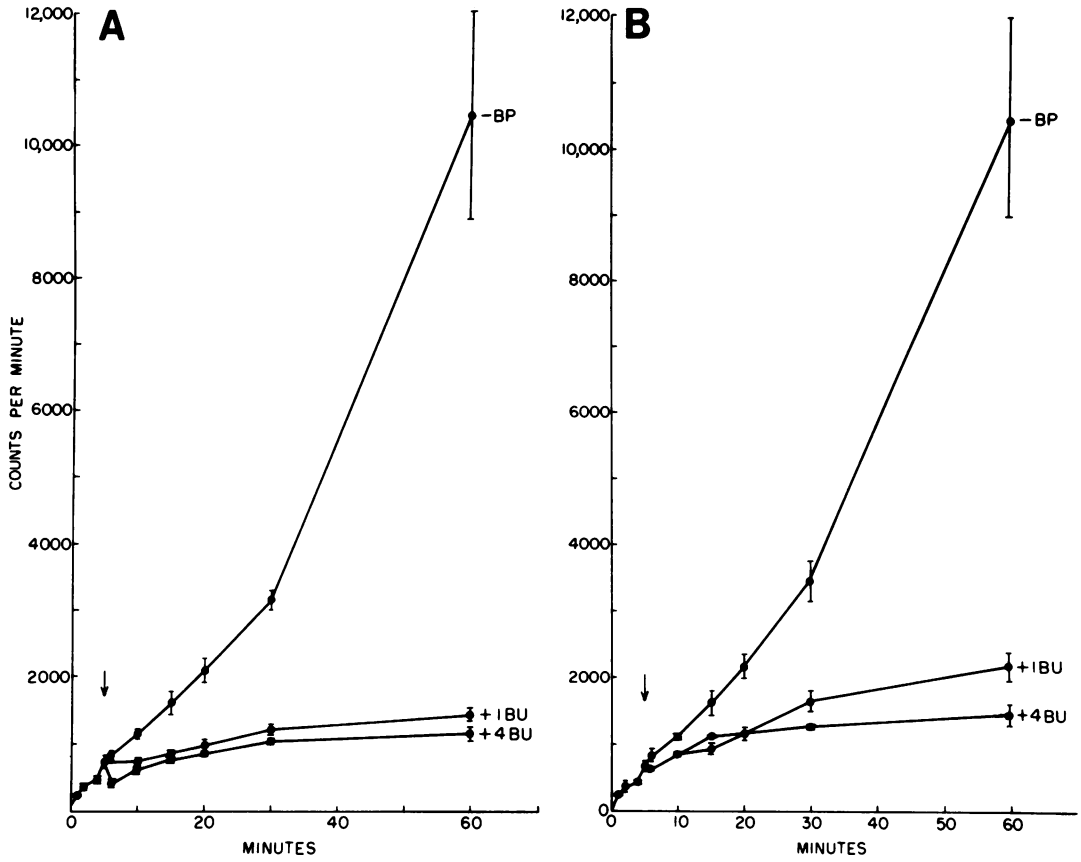

FIG. 1. Effect of BP on proline (A) uptake and (B) incorporation. For both assays, *P. aeruginosa* type I cells were allowed to accumulate [^{14}C]proline for 5 min and BP or buffer was added as indicated by the arrow. BP was added at the concentration required to kill 97 ± 2% of the bacteria present (1 BU) or at a fourfold multiple of that amount (4 BU). Proline transport (A) was measured by counting whole cells retained on Millipore filters and incorporation (B) was measured by counting TCA-precipitable counts retained on Millipore filters. Data points are mean values ± standard error, n = 3.

tories, Rockville Centre, N.Y.) column, which was eluted with a gradient of 0.1 to 0.7 M NaCl in CP buffer, pH 7.0. A pool of bactericidal fractions was concentrated by ultrafiltration, using a YM-10 membrane (Amicon Corp.). Protein concentration was determined by the Hartree modification of the Lowry method (3). This two-step purification scheme yielded a single 55,000-mlecular-weight band after 1 μg of protein was applied to sodium dodecyl sulfate-polyacrylamide gels for electrophoresis (7) and silver staining (11). Because the detection limit of this staining procedure in our hands was 1 ng, we have concluded that BP is at least 99.9% pure. Specific bactericidal activity was increased 150-fold, as determined by viable plate counts after dose-response of *P. aeruginosa* to BP. The amount of protein required to kill 95% of the bacterial inoculum of 5×10^6 was defined as 1 bactericidal unit (BU).

Amino acid uptake and incorporation. Uptake of amino acids by *P. aeruginosa* and *Proteus mirabilis* was measured by a modification of the method described by Uratani and Hoshino (15). A total of 5×10^6 CFU of bacteria per ml in CP-NB (10:1; pH 5.6) were aerated throughout the experiment on a reciprocal shaking water bath at 37°C. Cells were preincubated for 6 min, and at time zero 0.8 μCi of L-[U-^{14}C]proline or L-[U-^{14}C]leucine per ml was added. To measure amino acid uptake, 50-μl samples were removed, diluted with 5 ml of the suspension solution, filtered through membrane filters of 0.45 μm pore size (Millipore Corp., Bedford, Mass.), and washed once with 5 ml of the same solution. Five minutes into the assay, BP was added to the test cells or buffer was added to the control cells, and samples were removed at intervals for up to 1 h. The amount of proline or leucine incorporation into trichloroacetic acid (TCA)-insoluble fractions was determined by mixing 50 μl of the cell suspension with 5 ml of cold 10% TCA for 10 min at 4°C. Precipitates were trapped on Millipore filters and washed with 5 ml of 5% TCA. For both uptake and incorporation assays, membrane filters were air dried, immersed in 10 ml of scintillation fluid {19 mM PPO (2,5-diphenyloxazole)–0.29 mM POPOP [1,4-bis(5-phenyloxazolyl) benzene] in toluene}, and counted in a Beckman LS-7500 liquid scintillation counter.

Respiration measurements. Oxygen consumption was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Cells were suspended at 10^7 CFU/ml in CP-NB (2:1; final pH 5.8) in a total volume of 3 ml and stirred vigorously throughout the experiment. Temperature was maintained at 37°C. O_2 concentration in the closed reaction vessel was monitored from time zero by continuous recording and at 5 min BP, 30 mM $CaCl_2$, or 30 mM $MgCl_2$ was added. Consumption of O_2 was monitored for an additional 30 min.

ATP determination. Total cellular ATP was determined by firefly bioluminescence as described by Ludin and Thore (8). Firefly lantern extract-50 (FLE-50) was purchased from

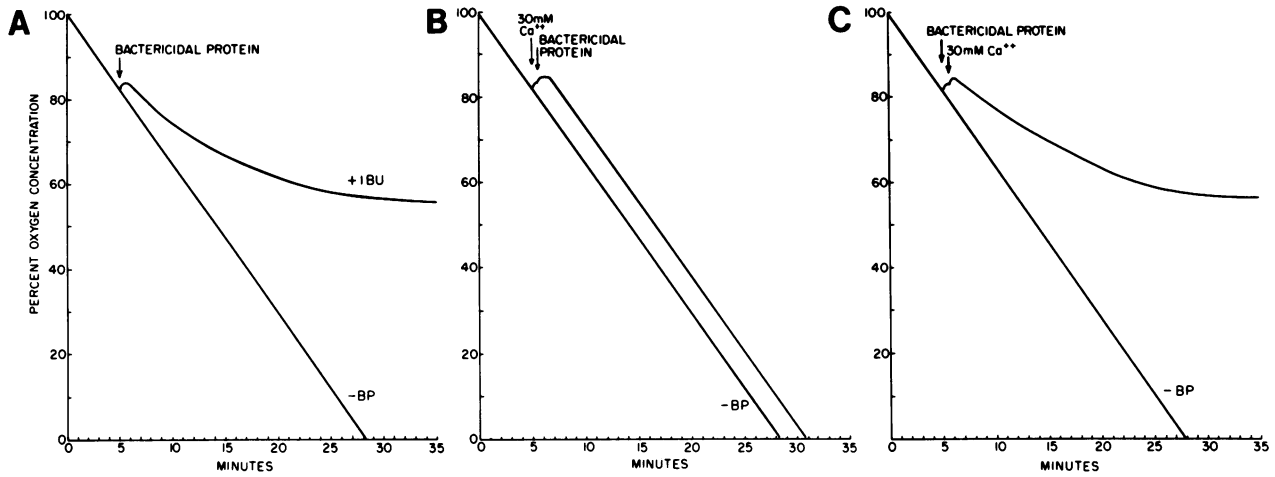

FIG. 2. Effect of BP on respiration. In the representative experiment shown, *P. aeruginosa* type I cells were incubated at 37°C for 5 min and (A) 1 BU of BP was added as indicated by the arrow, (B) 30 mM $CaCl_2$ was added, followed by BP, or (C) BP was added, followed by 30 mM $CaCl_2$. A total of 96 ± 2% of the cells were killed by the BP at 1 min after its addition (A and C), while cells were protected by the prior addition of Ca^{2+} (B) and thus were not killed by BP. The lower tracing in all three panels represents the respiration of *P. aeruginosa* type I not exposed to BP.

Sigma Chemical Co. (St. Louis, Mo.). A 50-mg portion of FLE-50 was suspended in 20 ml of 0.1% bovine serum albumin–10 mM $MgSO_4$–1 mM EDTA. Diluted FLE-50 was stored overnight at 4°C to allow endogenous luminescence to decay. Approximately 5×10^6 CFU of *P. aeruginosa* per ml in CP-NB (10:1; final pH 5.6) were exposed to BP at 37°C in a reciprocal water bath shaker. At timed intervals, 1-ml samples were removed and extracted for 5 min at room temperature after addition of 1 N perchloric acid. The pH was restored to 7.75 with 1 N KOH and 1.5 M Tris-EDTA buffer. Diluted FLE-50 (0.4 ml) was added to each 1-ml sample just before single photon counting in a Beckman LS-7500 liquid scintillation counter. Samples were counted for 2 min, and peak counts per minute were converted to moles of ATP with a standard curve derived from measurements of known amounts of ATP disodium salt (Sigma Chemical Co.).

Measurement of membrane potential. The membrane potential of cells was measured by the influx of [^{14}C]thiocyanate, a lipophilic anion, with the following modifications of the method of Uratani and Hoshino (15). Cells were concentrated to 10^9 CFU/ml, suspended in CP-NB (1:1; final pH 5.8), and preincubated at room temperature for 10 min. Potassium [^{14}C]thiocyanate (0.1 μCi/ml) and [^{3}H]inulin (1.0 μCi/ml) were added at time zero and BP or 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added at 1 min. At timed intervals, samples were removed and processed without further modification of the method (15).

Chemicals. All chemicals were of reagent grade. L-[U-^{14}C]proline (273 mCi/mmol) and L-[U-^{14}C]leucine (300 mCi/mmol) were purchased from Dupont New England Nuclear Research Products (Boston, Mass.). Potassium [^{14}C]thiocyanate (58 mCi/mmol) and [^{3}H]inulin (1.6 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

Unless otherwise indicated, purified BP was added to *P. aeruginosa* at the endpoint of 1 BU. One BU is that amount of protein required to kill 95% of 5×10^6 bacteria and ranged between 60 and 100 ng of protein. Bacteria exposed to BP remained intact, as determined by light microscopy and enumeration with a Petroff-Hausser counting chamber.

Effect of BP on amino acid uptake and incorporation. The effect of BP on cellular active transport and amino acid incorporation was examined with radiolabeled proline (Fig. 1) or leucine. Untreated *P. aeruginosa* type I accumulated proline at a constant rate which paralleled its incorporation into macromolecular protein represented by a TCA-insoluble fraction (Fig. 1A and B). The addition of 1 BU of BP caused an immediate and pronounced decrease in uptake of proline. At 4 BU, an initial efflux of free proline was seen at 6 min (Fig. 1A). Corresponding to the decrease in amino acid uptake, a rapid inhibition of protein synthesis occurred (Fig. 1B). Similar results were obtained with *E. coli* B, with *P. aeruginosa* incubated at pH 7.0, and with the substitution of L-[U-^{14}C]leucine for proline (data not shown). At concentrations of <1 BU, the decline in uptake and incorporation of amino acids was proportional to the percentage of bacteria killed. It was demonstrated with *Proteus mirabilis*, a bacterium resistant to BP, that amino acid uptake and incorporation were not affected by BP, although uptake and incorporation were readily stopped when 50 μM CCCP or 20 mM KCN was present (data not shown).

Effect of BP on respiration. The effect of BP on the respiration of *P. aeruginosa* type I was examined without and with 30 mM $CaCl_2$, added before or after BP (Fig. 2). Cells not exposed to BP respired at a constant rate for approximately 30 min and until the oxygen in the reaction vessel was depleted. The addition of BP to the cell suspension had an immediate effect of decreasing the rate of respiration, which continued to decline exponentially until respiration ceased completely at approximately 30 min after exposure to BP (Fig. 2A). At least 95% of the bacteria were dead, as determined by viable plate counts of samples removed 1 min after exposure to BP. The bactericidal activity of BP was inhibited in the presence of 30 mM $CaCl_2$, and 100% of the cells remained viable as shown by plate count. When *P. aeruginosa* cells were in the presence of Ca^{2+} their respiration rate remained constant and similar to that of the control cells (Fig. 2B). The order of addition of cation and BP was crucial to the protective effect of Ca^{2+}. If cells were exposed to BP, followed by 30 mM $CaCl_2$ within

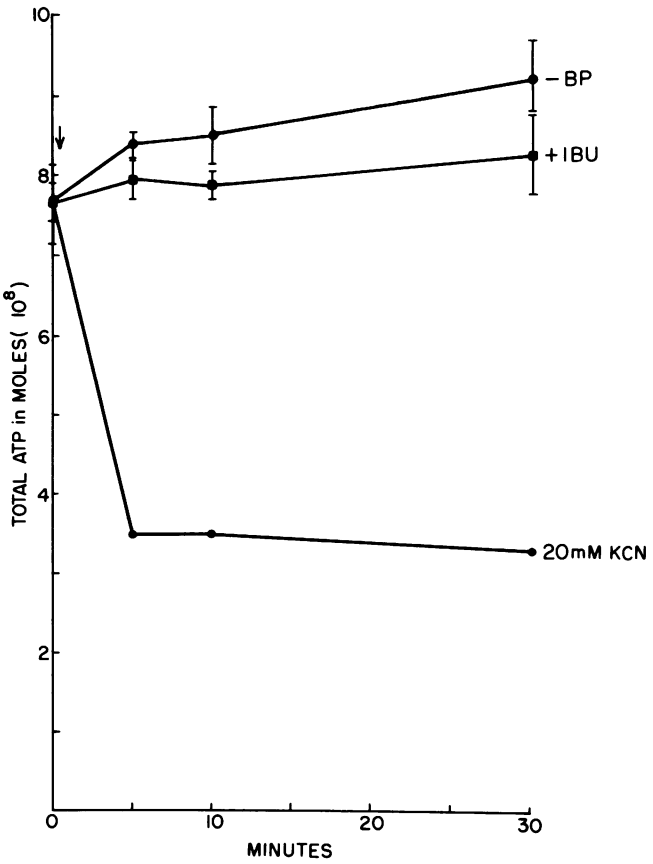

FIG. 3. Effect of BP on cellular ATP level. Total cellular ATP was measured before and after addition at 1 min of buffer (−BP), of 1 BU of BP (+BP), or of 20 mM KCN, as indicated by the arrow. *P. aeruginosa* type I concentration was at 5×10^6 CFU/ml, and 97 ± 2% of the cells were killed after exposure to BP. Data points represent mean values ± standard error, $n = 4$.

seconds of BP, 96 ± 2% of the cells were killed and respiration rates declined as if Ca^{2+} was not present (Fig. 2C). When 30 mM $MgCl_2$ was substituted for $CaCl_2$ the same results were obtained (data not shown). Similar results were observed with *P. aeruginosa* ATCC 27312 or *E. coli* B at pH 5.8 and with either *P. aeruginosa* strain incubated at pH 7.0 (data not shown).

Effect of BP on cellular ATP. The ATP pool of *P. aeruginosa* type I exposed to BP remained constant in spite of a 97 ± 2% loss of viability (Fig. 3). Control cell ATP levels rose slightly over the 30-min duration of the assay due to cell division. Total ATP of bacteria incubated with 20 mM KCN, a respiratory blocking agent, dropped within minutes. ATP levels of *P. aeruginosa* type I exposed to 1, 2, or 4 BU of BP remained constant for up to 2 h after >97% of the cells were killed (data not shown). Following Millipore filtration of samples, filtrates were shown to contain no measurable ATP (data not shown), indicating that the ATP detected in this assay was entirely intracellular and had not leaked from the cells treated with BP.

Effect of BP on membrane potential. Respiring bacteria that maintain a membrane potential accumulate only very small amounts of thiocyanate (SCN^-) anion in their cytoplasmic space (15). Membrane depolarization is accompanied by the influx of SCN^-, a lipophilic anion. Although [^{14}C]SCN^- was excluded by untreated *P. aeruginosa* type I in our experiments at 37°C, the cell associated ^{14}C counts per minute were approximately 25% higher than when cells were incubated at 24°C. Thus, our experiments were carried out at 24°C and the concentration of SCN^- inside and outside of the cells was estimated by comparing the ratio of cell-associated SCN^- to inulin. [^{3}H]inulin counts served as a monitor of the extracellular space and remained relatively constant throughout each experiment. The addition of 50 μM CCCP, an uncoupler of oxidative phosphorylation, caused a partial membrane depolarization, as indicated by accumulation of SCN^- anion in the cytoplasmic space. Cells killed with BP accumulated the SCN^- anion in a manner similar to the cells exposed to CCCP. The ratio of [^{14}C]SCN/[^{3}H]inulin rose from 0.26 to 0.37 for cells exposed to BP and from 0.26 to 0.40 for cells depolarized by CCCP (Fig. 4A). Control cells showed only a slight increase in ratio from 0.26 to 0.31. Although the cytoplasmic membrane potential of *P. aeruginosa* type I dropped with exposure to both BP and 50 μM CCCP, it did not totally dissipate. Under the same experimental conditions, the membrane of rough strain *E. coli* B was completely depolarized within minutes of addition of 50 μM CCCP or within 10 to 20 min of addition of 1 BU or BP (Fig. 4B). Under these conditions of decreased temperature, killing of bacteria did not reach 95% until 10 min after exposure to BP.

DISCUSSION

BP purified from normal PMN is a 55,000-molecular-weight protein with amino acid composition, molecular weight, bactericidal potency, and bactericidal specificity (Hovde and Gray, in preparation) which are closely similar to those of two proteins described in the literature. Weiss et al. isolated a 58,000 to 60,000-molecular weight protein from chronic myelogenous leukemia cells with greatest bactericidal activity against rough strains of *Salmonella typhimurium* or *E. coli* (17). They refer to this protein as bactericidal/permeability increasing protein (B/PI). The second protein, as reported by Shafer et al., is a 57,000-molecular-weight protein from chronic myelogenous leukemia cells with a similar microbicidal specificity and is referred to as cationic antimicrobial protein (CAP) (13). A physiological mechanism of action for cationic antimicrobial protein towards *S. typhimurium* and *E. coli* has not been reported, but Weiss et al. have reported that B/PI creates a discrete outer membrane permeability change (17). Bactericidal and permeability-increasing activities of B/PI were both inhibited by Ca^{2+} or Mg^{2+} (17). The authors suggest that the alteration in membrane permeability may be a physiological lesion leading to death of these microorganisms (1, 17). They also report that there is no significant change in bacterial macromolecular synthesis for up to 2 h after irreversible loss of the ability to form colonies (1, 17).

The effect of the BP on *P. aeruginosa* is strikingly different from that of B/PI on *S. typhimurium* and *E. coli*. We have observed dramatic alterations in amino acid transport, respiration, and membrane potential within minutes of exposure of *P. aeruginosa* to BP. We believe that bacterial death is the consequence of a biochemical lesion of the cytoplasmic membrane, specifically, a lesion causing membrane depolarization. The physiological studies done by Weiss et al. (17) were carried out at pH 7.0, the reported optimum for B/PI. In an effort to explore the differing effects of BP and B/PI, some of our experiments were repeated at pH 7.0. The amino acid transport and incorporation studies and the respiration studies at pH 7.0 showed the same rapid and dramatic changes of BP-treated *P. aeruginosa* seen under more acidic conditions. Other differences in composition of

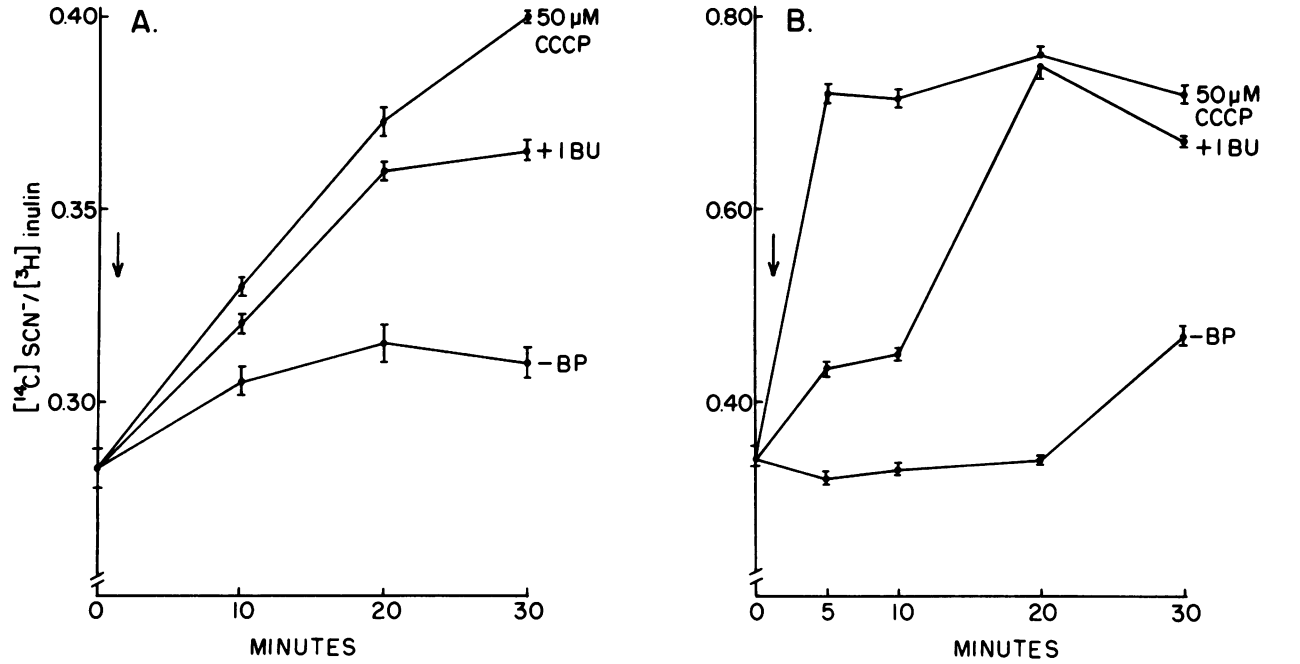

FIG. 4. Effect of BP on membrane potential. Influx of thiocyanate anion was measured as a ratio of cell-associated [^{14}C]SCN$^-$ to [^{3}H]inulin. One minute following equilibration at 24°C of 10^9 CFU of *P. aeruginosa* type I (A) or *E. coli* B (B) per ml, additions of buffer (−BP), BP (1 BU), or 50 μM CCCP were made, as indicated by the arrow. Cell death was 96 ± 3% at 10 min after addition of BP. Data points represent mean values ± standard error, $n = 3$.

incubation media would not appear to account for the divergent results. One might also argue that the different results are merely a reflection of the differences between the microorganisms studied by the two laboratories. However, we found an even more profound difference when rough strain *E. coli* B was substituted for *P. aeruginosa* in our membrane depolarization experiments (Fig. 4B). The effect of BP on respiration and amino acid incorporation by *E. coli* B and *P. aeruginosa* was similar. Thus, we cannot explain the very different effects of BP and B/PI at this time.

P. aeruginosa is an aerobic organism which derives all of its energy from oxidative phosphorylation. According to the chemiosmotic theory of Peter Mitchell, the driving force in maintaining ATP in an aerobic organism is the membrane potential which is composed of both a chemical and an electrical gradient across the membrane (9). Dissipation of this potential can be associated with the physiological changes we have observed with exposure to BP. Respiration and active transport would both cease and, without uptake of amino acids, protein synthesis would also stop. It was, however, surprising to see no change in ATP levels. Theoretically, one would expect that ATP levels would drop rapidly as the membrane potential is dissipated. The simplest explanation for our result is that ATP is neither synthesized nor utilized by BP-treated *P. aeruginosa*. Protein synthesis, the metabolic function requiring the most energy, has all but ceased and it may be that ATP levels remain constant because the cells are "paralyzed" in terms of both ATP synthesis and utilization.

The time course of change in membrane potential is of particular interest in assessing whether it is a cause or an effect of cell death. We believe that it is an early event after BP binds to the cells. The experiments with sensitive *E. coli* B (rough strain) showed that membrane potential drops to zero within minutes of death due to BP. The membrane potential experiments were done at a lower temperature (24 versus 37°C) to enhance thiocyanate exclusion by untreated bacteria. It appears that delayed and partial depolarization is a perculiarity of *P. aeruginosa* at the lower temperature of this assay. The crucial point is that BP depolarized the membrane at a similar rate and to a similar extent as did CCCP, a known depolarizing agent.

The pattern of physiological changes seen in *P. aeruginosa* as it is killed by purified BP supports a hypothesis that the mechanism of action of this natural "antibiotic" is one of cytoplasmic membrane damage. The actual process by which BP creates membrane depolarization has not yet been explored, but it may be similar to the action of some colicins. Colicin E1 and Ia are 56,000 and 79,000-molecular-weight proteins, respectively, and are synthesized by certain plasmid-harboring *E. coli* (6). When sensitive *E. coli* are killed, the evidence suggests that these colicins form an ion-permeable channel in the bacterial cytoplasmic membrane which leads to dissipation of membrane potential (2). *E. coli* killed by colicin E1 display physiological changes similar to the effects of BP on *P. aeruginosa* (2). Interestingly, increased outer membrane permeability is a consequence of membrane depolarization due to colicin E1 (2). While this may also explain the outer membrane permeability changes described by Weiss et al. (17), it does not reconcile the striking differences observed in the effects of BP and B/PI on protein synthesis. We hope to clarify this discrepancy by examining the *E. coli* O111:B4 and *S. typhimurium* 395MS strains studied by these authors (16). It is with great curiosity that we speculate about human PMN synthesis of a BP which resembles some *E. coli* colicins in both size and mode of action. Studies to investigate the extent of the similarities are planned.

ACKNOWLEDGMENTS

C.J.H. was supported by a Public Health Service training grant (5T32-HLI 07114) from the National Heart, Lung and Blood Institute

and by a fellowship from the Samuel Roberts Noble Foundation, Ardmore, Okla.

We are grateful to Y. Uratani for his valuable advice regarding measurement of bacterial membrane potential.

LITERATURE CITED

1. **Elsbach, P., and J. Weiss.** 1983. A reevaluation of the roles of the O_2-dependent and O_2-independent microbicidal systems of phagocytes. Rev. Infect. Dis. **5**:843–853.
2. **Gould, M. J., and W. A. Cramer.** 1977. Studies on the depolarization of *Escherichia coli* cell membrane by colicin E1. J. Biol. Chem. **252**:5491–5497.
3. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422–427.
4. **Holmes-Gray, B.** 1977. Metabolic stimulation and bactericidal function of polymorphonuclear leukocytes. Res J. Reticuloendothel. Soc. **22**:87–88.
5. **Klebanoff, S. J.** 1980. Myeloperoxidase-mediated cytotoxic systems, p. 279–308. *In* A. J. Sbarra, and R. D. Strauss (ed.), The reticuloendothelial system. A comprehensive treatise, vol. 2. Plenum Press, New York.
6. **Konisky, J.** 1982. Colicins and other bacteriocins with established modes of action. Annu. Rev. Microbiol. **36**:125–144.
7. **Laemmli, U. K.** 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T_4. Nature (London) **227**:680–685.
8. **Ludin, A., and A. Thore.** 1975. Analytical information obtainable by evaluation of the time course of firefly bioluminescence in the assay of ATP. Anal. Biochem. **66**:47–63.
9. **Mitchell, P.** 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature (London) **191**:144.
10. **Neu, H. C.** 1983. The role of *Pseudomonas aeruginosa* in infections. J. Antibiot. Chem. **11**(Suppl. B):1–13.
11. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensative silver stain for detecting protein in polyacrylamide gels. Anal. Biochem. **105**:361–363.
12. **Peterson, P. K.** 1980. Host defense against *Pseudomonas aeruginosa*, p. 103–118. *In* L. P. Sabath (ed.), *Pseudomonas aeruginosa*—the organisms, diseases it causes and their treatment. E. Huber, Vienna.
13. **Shafer, W. M., L. E. Marlin, and J. K. Spitznagel.** 1984. Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. Infect. Immun. **45**:29–35.
14. **Spitznagel, J. K.** 1984. Nonoxidative antimicrobial reactions of leukocytes. Contemp. Top. Immunobiol. **14**:283–328.
15. **Uratani, Y., and T. Hoshino.** 1984. Pyocin R1 inhibits active transport in *Pseudomonas aeruginosa* and depolarizes membrane potential. J. Bacteriol. **157**:632–636.
16. **Weiss, J., S. Beckerdite-Qualiata, and P. Elsbach.** 1980. Resistance of gram-negative bacteria to purified bactericidal leukocyte proteins. J. Clin. Invest. **65**:619–628.
17. **Weiss, J., P. Elsbach, I. Olsson, and H. Odeberg.** 1978. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J. Biol. Chem. **253**:2664–2672.
18. **Young, L. S., and D. Armstrong.** 1972. Human immunity to *P. aeruginosa*. 1. In vitro interaction of bacteria, polymorphonuclear leukocytes and serum factors. J. Infect. Dis. **126**:257.